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Developing potent BTK^{C481S} PROTACs for ibrutinib-resistant malignant lymphoma

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ABSTRACT

Ibrutinib is a first-line treatment drug for B-cell malignancies. However, resistance to ibrutinib has been reported due to BTK^{C481S} mutation. Although PROTAC strategy is expected to overcome this clinical resistance, it has limitations such as large molecular weight and moderate bioactivity, which restrict its potential clinical application. Herein, we report a new type of potent BTK^{C481S}-targeting PROTAC degrader. Through design, computer-assisted optimization and SAR studies, we have developed a representative BTK^{C481S} degrader **L6** with a much smaller molecular weight and improved solubility. Notably, **L6** demonstrates better BTK degrading activity and lower IC_{50} value in ibrutinib-resistant cell line than the first-generation BTK degrader **P13I**. Optimization strategy of **L6** provides a general approach in the development of PROTACs targeting BTK and other proteins for future study.

Non-Hodgkin's lymphoma (NHL) is the most common hematological malignancy in adults. Approximately 4.3 million people worldwide suffer from this disease [1]. Diffuse large B-cell lymphoma (DLBCL) is the most common B-cell non-Hodgkin's lymphoma (B-NHL), accounting for roughly 30% [2,3]. Bruton's tyrosine kinase (BTK), as a member in BCR signaling pathway, plays a key role in the development, differentiation, survival, and signaling of B cells [4,5]. Therefore, signal transduction of BTK is crucial for the survival of B-cell leukemia and lymphoma [6,7].

Ibrutinib is the first oral BTK inhibitor approved by the FDA, which is used as a first-line treatment drug for various lymphomas, including CLL, MCL, FL, *etc.* [8]. Although ibrutinib has remarkable therapeutic effect, clinically drug-resistant cases still appeared with poor prognosis. The main mechanism of ibrutinib-resistance is BTK^{C481S} mutation, which can also lead to the failure of other BTK covalent inhibitors, such as zanubrutinib and acalabrutinib [9,10]. Thus, there is an urgent need to develop new effective agents against BTK mutations.

Proteolysis-targeting chimeras (PROTAC) has recently become a research hot spot in academia and pharmaceutical industry [11,12]. However, PROTAC molecule has several intrinsic limitations, such as large molecular weight (roughly 900–1000 g/mol), lengthy linker [13]. These shortcomings may hinder further applications of PROTAC in clinical trials [14]. In 2018, we reported the first-generation of BTK degrader **P13I** [15,16]. However, **P13I** has a relatively large molecular weight with poor water solubility and moderate bioactivity. How to design the new structure of PROTACs while improving its bioactivity is a very challenging task.

In this study, we developed a new type of BTK^{C481S} degrader **L6** through computer-assisted design. Compared with **P13I**, molecular weight of **L6** was reduced about 200, and the solubility and predicted permeability of **L6** were improved. Moreover, the novel BTK degrader **L6** has better bioactivity than **P13I** in ibrutinib-resistant BTK^{C481S} HBL-1 cells (Fig. 1). In addition, we also conducted SAR study *via* molecular docking, providing a general procedure for further optimization of PROTACs.

In order to optimize the structure and bioactivity of BTK degrader, we first designed derivatives of **P13I** with different E3 ligands and linkers. Then, ACD/Labs was employed for analysis. The results indicated that, BTK degraders with lenalidomide ligand could have better CaCo-2 cell membrane permeability than those with pomalidomide ligand (Table 1).

Based on the analysis results above, we conducted the solubility test after synthesis of PROTACs (Scheme 1) [15]. Compound **mL13I**

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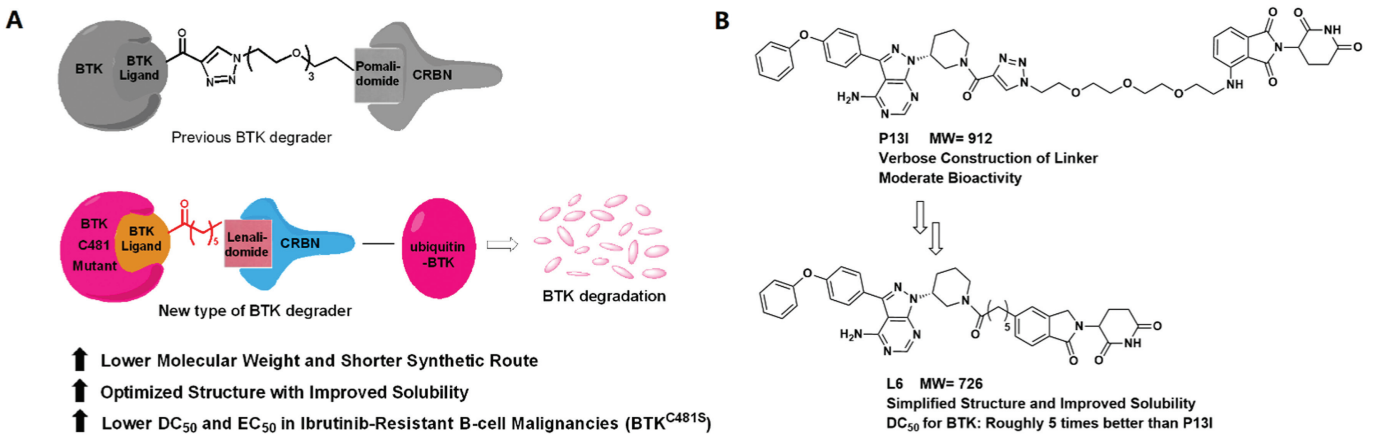


Fig. 1. Development of novel PROTACs targeting ibrutinib-resistant BTK^{C481S}. (A) Schematic representation and breakthrough of compound **L6**. (B) Design and evolutionary route of novel BTK degraders.

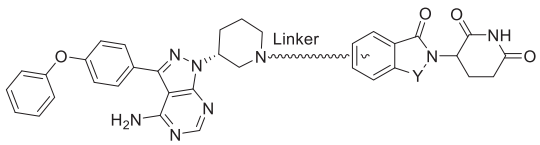
demonstrated a much better solubility than that of **mP13I**, indicated that lenalidomide ligand may also help to improve the solubility (Table 1). This phenomenon can also be found in compound **L9I** (Table 1). Therefore, lenalidomide was utilized as the E3 ligand moiety for BTK degrader development.

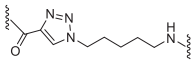
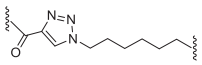
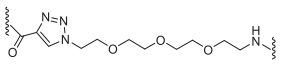
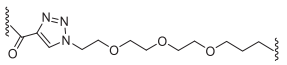
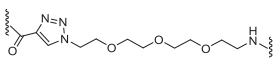
To further simplify the chemical structure of PROTACs, different BTK degraders with aliphatic linkers were prepared, and human Ramos cells and Mino cells were used to evaluate the degradation activity (Figs. S5 and S6 in Supporting information).

After the screening, we found that compound **L6** with 6 carbon linker and lenalidomide ligand had the best BTK degradation activity at concentrations of 11, 33 and 100 nmol/L (Fig. 2 and Table S1 in Supporting information). Compared with **P13I**, **L6** has a molecular weight reduction of about 200, and both hydrogen bond acceptors and rotatable bonds of **L6** are reduced by 8. Additionally, the water solubility of **L6** was about 3 times higher than that of **P13I**, and the predicted apparent permeability coefficient (Papp) of **L6** in Caco-2 cells increased by nearly 10 times (Table 1).

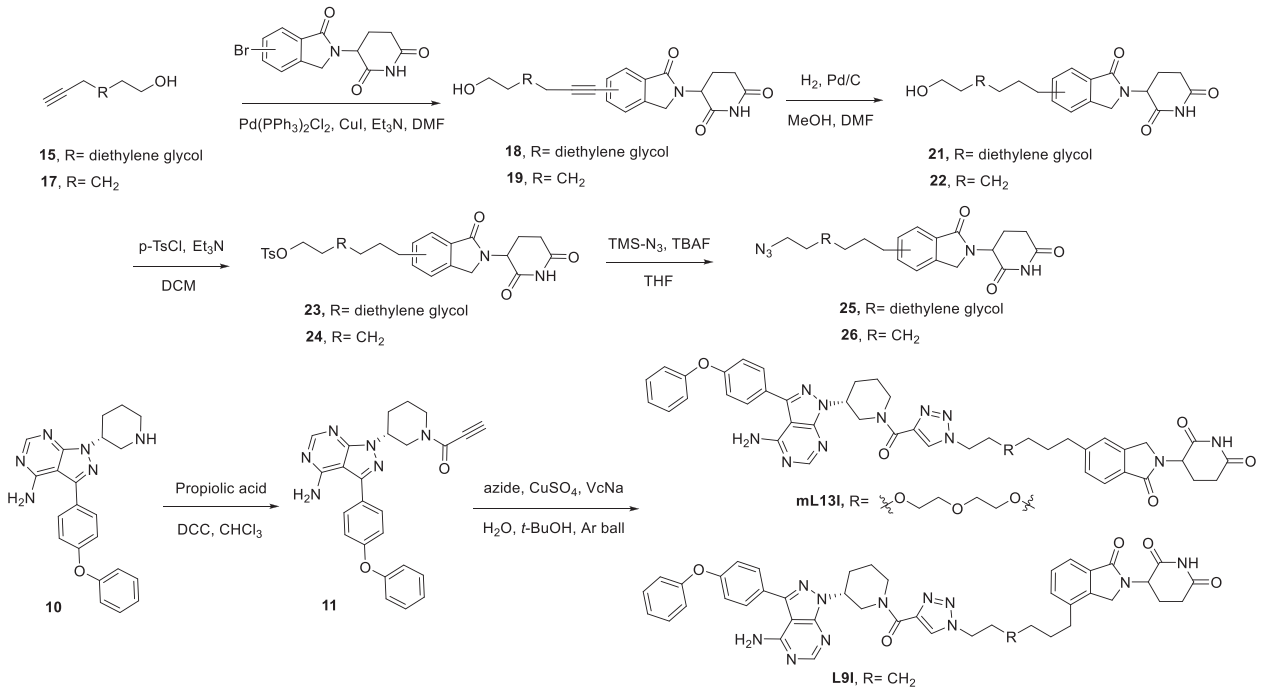
For the synthesis of compounds **mL13I** and **L9I** (Scheme 1), the Sonogashira coupling reaction was conducted with compound **15** and **17** as substrate to prepare compounds **18** and **19**. After reduction of alkynyl group, compound **21** and **22** were generated

Table 1
Chemical structures and parameters of novel BTK degraders and reference compound.



Compound	Linker	Y	Position of linker relative to Y	MW (g/mol)	H-Acceptors[a]	H-Donors[b]	Rot. bonds	Sol.[c]	CaCo-2 Papp (nm/s)[d]
L6		CH_2	*meta*	726	13	3	11	A	1150
L7		CH_2	*meta*	740	13	3	12	A	940
L8		CH_2	*meta*	754	13	3	13	A	530
P9I		CO	*ortho*	822	18	4	13	D	390
L9I		CH_2	*ortho*	807	16	3	13	A	1120
mP13I		CO	*meta*	912	21	4	19	E	100
mL13I		CH_2	*meta*	897	19	3	19	B	840
P13I		CO	*ortho*	912	21	4	19	E	130

[a] Hydrogen bond acceptor.
[b] Hydrogen bond donor.
[c] Compound was dissolved in DMSO:castor oil:PBS (1:1:8) system. A: 6.5–7.5 g/L. B: 5.5–6.5 g/L. C: 4.5–5.5 g/L. D: 3.5–4.5 g/L. E: 2.5–3.5 g/L.
[d] ACD/Labs (Advanced Chemistry Development, Inc.) predicted.



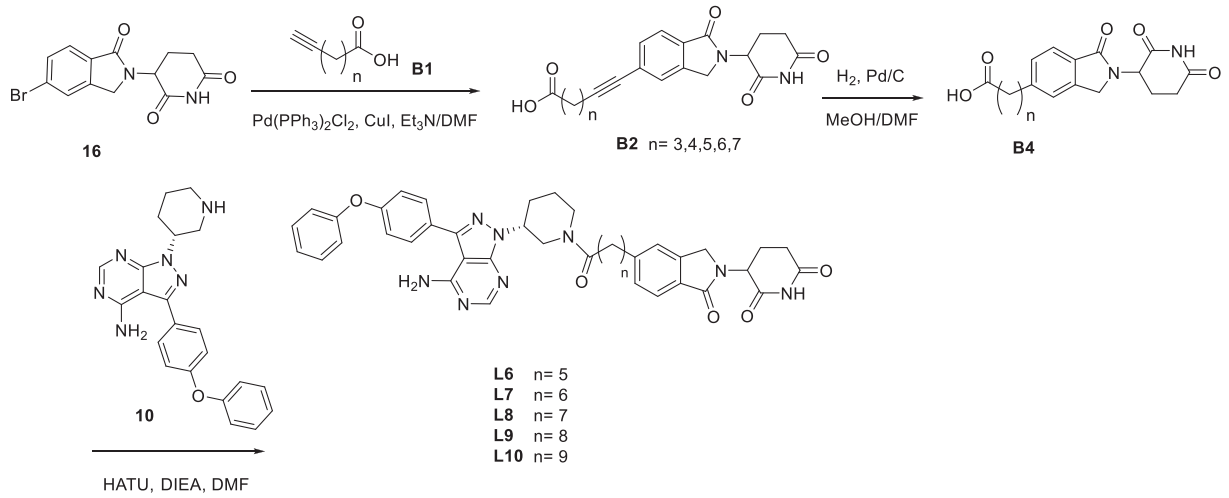
Scheme 1. Synthesis of compounds **mL13I** and **L9I**.

in which the hydroxy group was protected by *p*-toluenesulfonyl group. Compounds **23** and **24** were then utilized in substitution reaction to prepare compound **25** and **26**. After the condensation reaction, ibrutinib derivatives were produced and then transformed to **mL13I** and **L9I** *via* click reaction in the last synthetic step. **P13I** and its derivatives were prepared as before [15]. For the synthesis of **L6-L10** (Scheme 2), the intermediates **B2** were synthesized *via* a Sonogashira coupling which was followed by the reduction of alkynyl group to generate alkyl acid ligand. Finally, compounds **B4** were condensed with ibrutinib precursor to prepare the amide derivative as the target compounds **L6-L10**. Compared with **P13I, mL13I, L9I** and their derivatives, **L6** has a pretty short synthetic route, which brings convenience for large-scale preparation in the future.

In order to investigate the conformational differences between **L6** and **P13I** in BTK-degrader-CRBN ternary complex, compounds were docked into BTK and CRBN structures. As showed in Fig. S10 (Supporting information), binding positions of **L6** in two proteins were consistent with the crystal structures. Linker of **P13I** was curved, while the aliphatic linker of **L6** was almost straight.

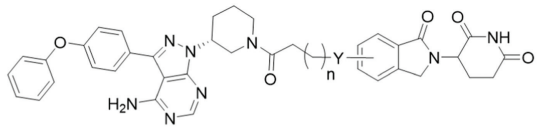
Polyethylene glycol linker may easily bind to H_2O molecules. Therefore, the protein-protein interaction could be hindered. However, compound **L6** is smaller and has fewer conformations; this may result in lower binding free energy and better stability of the ternary complex. Thus, efficiency of ubiquitination-degradation could be increased. To further confirm this phenomenon in experiments, we synthesized **L7d** with polyethylene glycol linker as derivative of **L7**. The results showed that bioactivity of **L7d** was reduced (Figs. S4 and S8 in Supporting information).

Next, to further evaluate the bioactivity of compound **L6**, different lymphoma cell lines were used. The data showed that **L6** could efficiently degrade BTK protein in Ramos cell line



Scheme 2. Synthesis of compounds **L6-L10**.

A



Compound	OL6Y	OL7Y	OL8Y	OL9Y	L6Y	L7Y	L8Y	L9Y	OL6	OL7	OL8	OL9	L6	L7	L8	L9
Y																
n	2	3	4	5	2	3	4	5	2	3	4	5	2	3	4	5
Position of Y relative to methylene	*ortho*	*ortho*	*ortho*	*ortho*	*meta*	*meta*	*meta*	*meta*	*ortho*	*ortho*	*ortho*	*ortho*	*meta*	*meta*	*meta*	*meta*

B

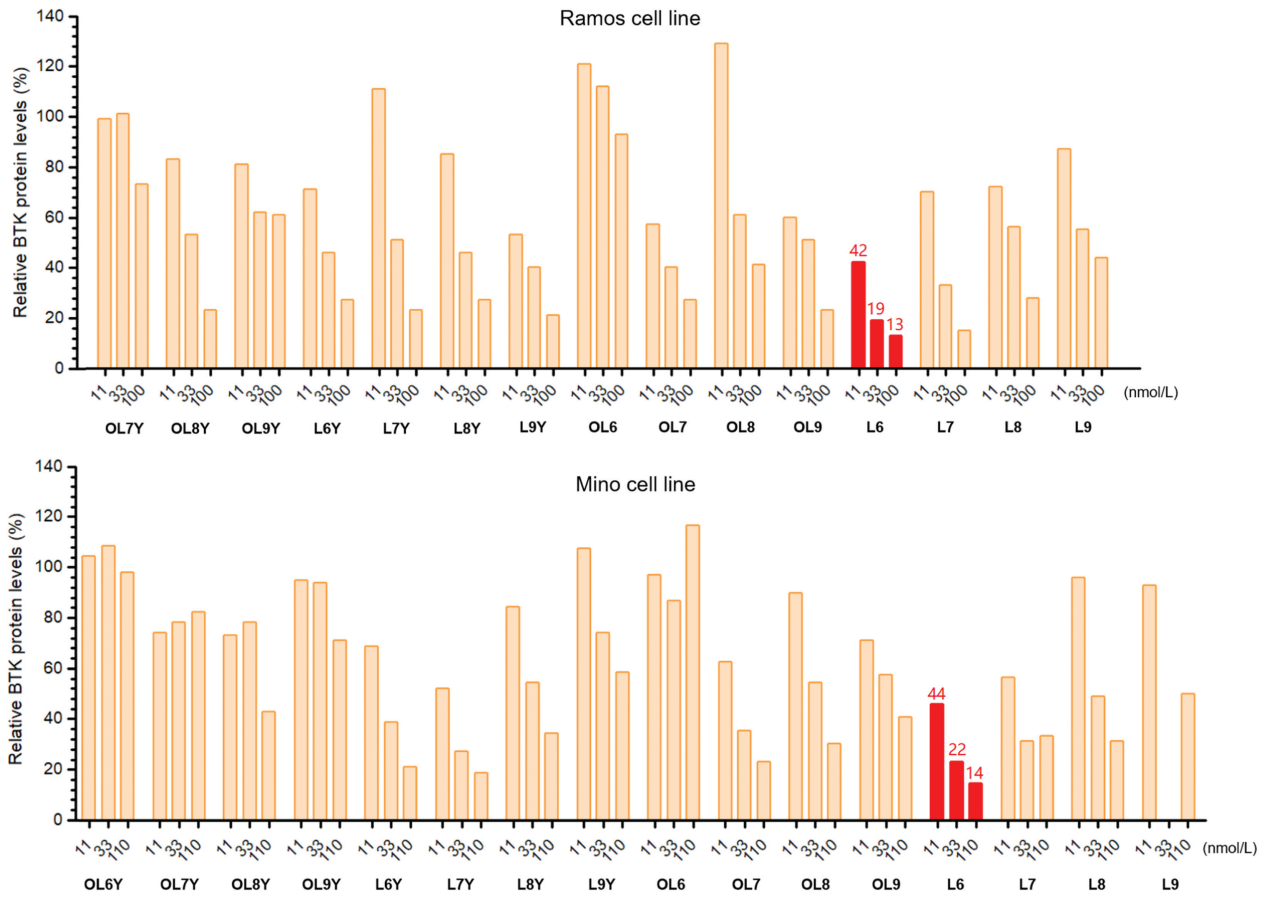


Fig. 2. Structures and degradation efficiency of novel PROTACs with aliphatic linkers in B-cell malignancy Mino cells and Ramos cells. (A) Structures of the new generation of PROTAC molecules targeting BTK. (B) Immunoblotting analysis of BTK protein and β-actin protein from Ramos cells or Mino cells treated with compounds for 48 h. In 12-well plates, 2×10^5 cells were incubated in each well at 37 °C. Grayscale analysis data was generated by ImageJ for the calculation of relative level of BTK protein.

($DC_{50} = 3.8$ nmol/L, Fig. 3), which was nearly 5 times stronger than that of **P13I** (Figs. 4A and B). The inhibitory activity of **L6** in ibrutinib-resistant BTK^{C481S} HBL-1 cells was also better than that of **P13I**. Compared with ibrutinib, the EC_{50} of **L6** was roughly 31 times better. Ibrutinib had nearly no efficacy to the mutant cell line (Fig. 4C and Table S2 in Supporting information) [10,17]. Therefore, **L6** as a PROTAC molecule cannot only overcome the failure of ibrutinib caused by the BTK^{C481S} mutation, but also improve the bioactivity of degraders.

To further evaluate the general toxicity of compound **L6**, BTK-insensitive DOHH2 cell line was utilized. As showed in Fig. 4D, **L6** had nearly no inhibitory activity even at 5000 nmol/L, indicated that **L6** may serve as a safe and effective BTK degrader.

In summary, a novel ibrutinib-resistant BTK^{C481S} degrader **L6** with high efficiency and solubility was developed *via* computer-assisted optimization. Compound **L6** has the lowest molecular weight among the current reported BTK degraders and better solubility than **P13I**. Compound **L6** has fewer hydrogen bond donors/acceptors and rotatable bonds, and the predicted Papp in Caco-2 cells increased nearly 10 times compared with that of **P13I**. In terms of bioactivity, the newly developed **L6** had a nearly 5-times increase in the degradation activity of BTK, and the inhibitory activity on ibrutinib-resistant BTK^{C481S} HBL-1 cells was stronger than **P13I**. EC_{50} of **L6** was about 31 times stronger than ibrutinib. Therefore, **L6** not only represents an effective degrader with improved bioactivity to overcome the failure of ibrutinib, but also optimizes the structure and synthetic route of BTK PROTACs. Lastly, SAR study showed that the aliphatic linker in **L6** had several advantages compared with polyethylene glycol linker of **P13I**, which provides a general and useful method for future development and optimization of PROTACs.

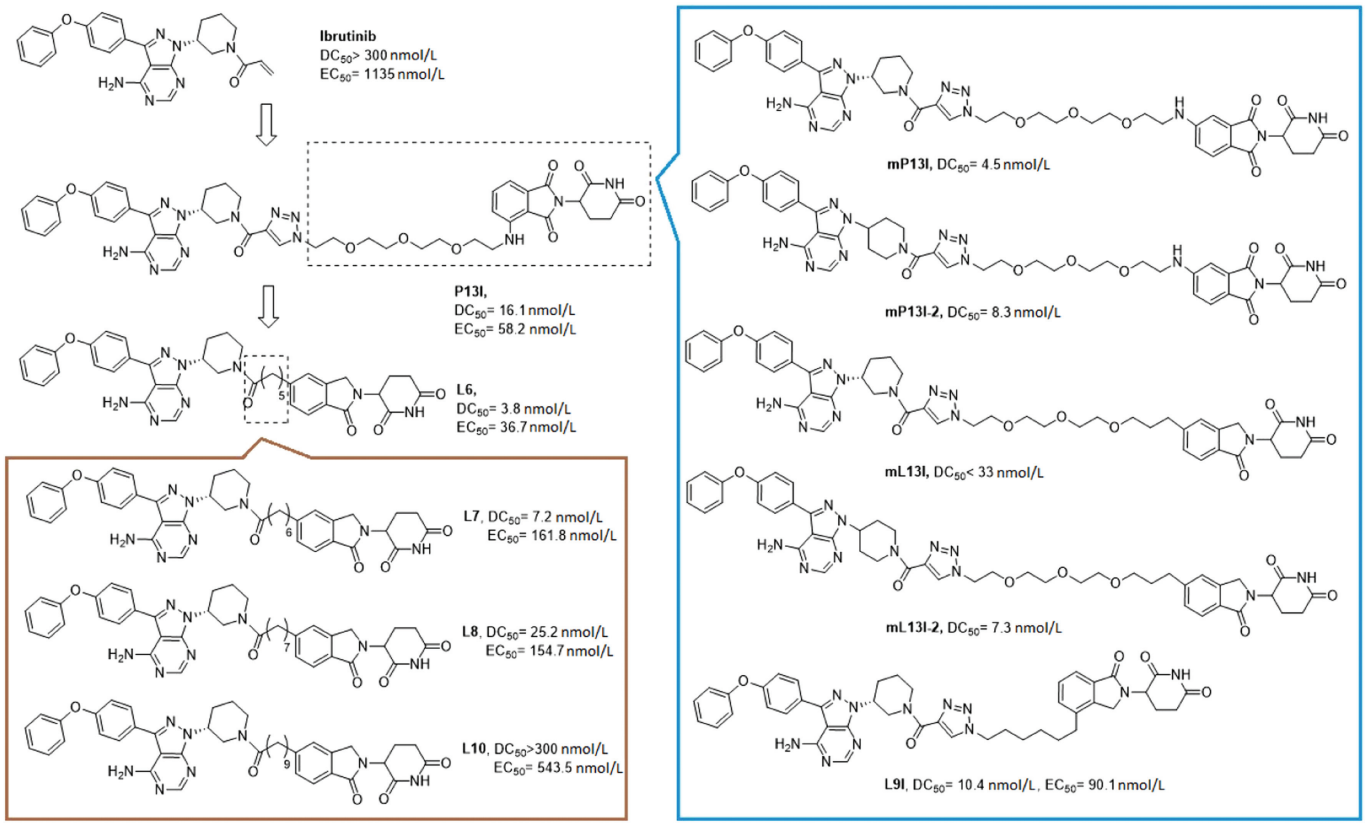


Fig. 3. DC_{50} values of Western blotting analysis in B-cell malignancy Ramos cells and EC_{50} values of cell growth inhibition in ibrutinib-resistant HBL-1 (BTK^{C481S}) cells. Immunoblotting analysis of BTK protein and β-actin protein from Ramos cells treated with compounds for 48 h. In 12-well plates, 2×10^5 cells were incubated in each well at 37 °C. DC_{50} data was generated by GraphPad Prism 5. For cell viability assays, HBL-1 (BTK^{C481S}) cells in 96-well plate were incubated for 72–96 h (3000 cells per well). The final EC_{50} was generated by MTT and GraphPad Prism 5.

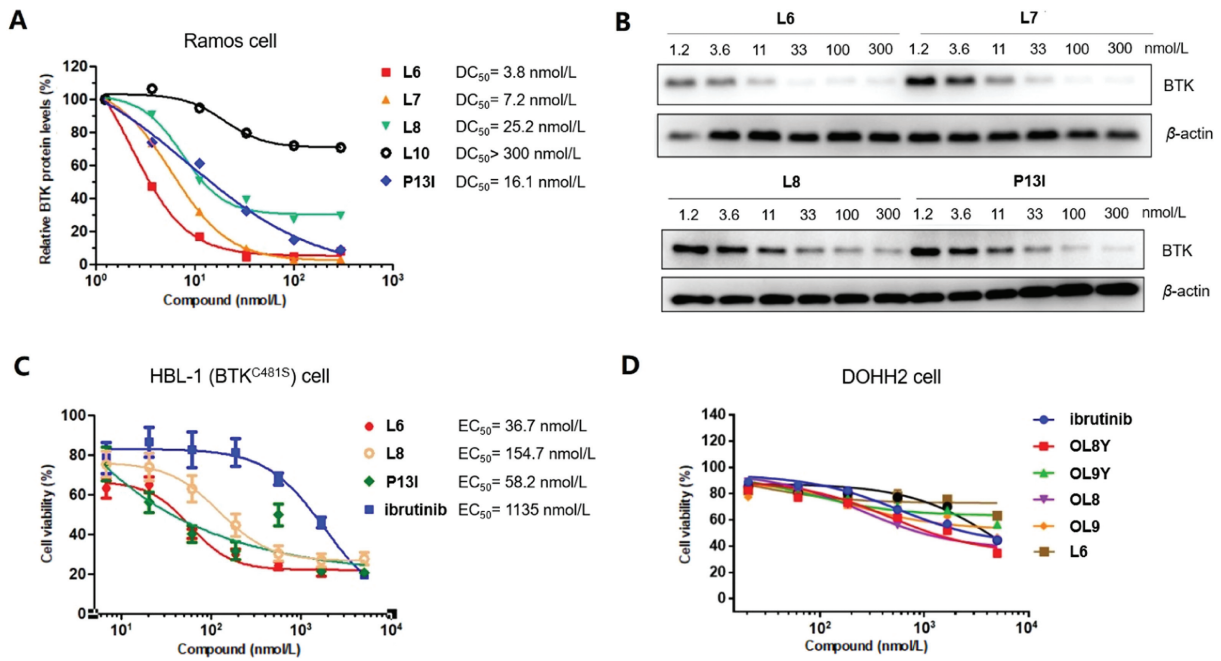


Fig. 4. Bioactivities of novel BTK^{C481S} degraders and reference compounds. (A) Relative protein level curves of BTK after treatment of Ramos cells with the indicated concentrations of compounds. (B) Immunoblotting analysis for BTK protein and β-actin protein from Ramos cells treated with the indicated concentrations of **L6**, **L7**, **L8** and **P13I** for 48 h. (C) For cell viability assays, HBL-1 (BTK^{C481S}) cells in 96-well plate were incubated for 72–96 h (3000 cells per well). The final EC_{50} was generated by MTT and GraphPad Prism 5. (D) For cell viability assays, DOHH2 cells in 96-well plate were incubated for 72–96 h (3000 cells per well). The final EC_{50} was generated by MTT and GraphPad Prism 5.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2022.107924.

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